Korean J Physiol Pharmacol
Vol 2: 101−108, February, 1998

Discrepancy between in vitro and in vivo Effect of $G\alpha_s$ Gene Mutation on the mRNA Expression of TRH Receptor

Seungjoon Park[1], Inmyung Yang[2], Sungvin Yim[1], Jooho Chung[1], Jeechang Jung[1], Kyechang Ko[1], Youngseol Kim[2], and Youngkil Choi[2]

[1]Department of Pharmacology and [2]Division of Endocrinology, Department of Internal Medicine, Kyunghee University College of Medicine and Institute of Genetic Engineering at Kyunghee University, Seoul 130−701, Korea

We investigated the effect of α-subunit of the stimulatory GTP-binding protein ($G\alpha_s$) gene mutation on the expression of the thyrotropin-releasing hormone (TRH) receptor (TRH-R) gene in GH3 cells and in growth hormone (GH)-secreting adenomas of acromegalic patients. In the presence of cyclohexicmide, forskolin and isobutylmethylxanthine, cholera toxin, and GH-releasing hormone (GHRH) decreased rat TRH-R (rTRH-R) gene expression by about 39%, 43.7%, and 46.7%, respectively. Transient expression of a vector expressing mutant-type $G\alpha_s$ decreased the rTRH-R gene expression by about 50% at 24 h of transfection, whereas a wild-type $G\alpha_s$ expression vector did not. The transcript of human TRH-R (hTRH-R) gene was detected in 6 of 8 (75%) tumors. Three of them (50%) showed the paradoxical GH response to TRH and the other three patients did not show the response. The relative expression of hTRH-R mRNA in the tumors from patients with the paradoxical response of GH to TRH did not differ from that in the tumors from patients without the paradoxical response. Direct PCR sequencing of $G\alpha_s$ gene disclosed a mutant allele and a normal allele only at codon 201 in 4 of 8 tumors. The paradoxical response to TRH was observed in 2 of 4 patients without the mutation, and 2 of 4 patients with the mutation. The hTRH-R gene expression of pituitaty adenomsa did not differ between the tumors without the mutation and those with mutation. The present study suggests that the expression of TRH-R gene is not likely to be a main determinant for the paradoxical response of GH to TRH, and that $G\alpha_s$ mutation may suppress the gene expression of TRH-R in GH-secreting adenoma. However, a certain predisposing factor(s) may play an important role in determining the expression of TRH-R.

Key Words: TRH, G protein, Acromegaly, GH3

INTRODUCTION

The cDNA of human thyrotropin-releasing hormone receptor (hTRH-R) gene was recently cloned from a growth hormone (GH)-secreting pituitary adenoma (Yamada et al, 1993). The expression of hTRH-R gene may in part explain the mechanism of paradoxical response of GH to TRH that is observed in some patients with acromegaly (Irie and Tsushima, 1972).

On the other hand, it was reported that rat TRH-R (rTRH-R) mRNA levels were decreased by agents that elevate intracellular cAMP in GH3 cells (Fujimoto and Gershengorn, 1991). A subset of human GH-secreting pituitary tumors has somatic mutations that encodes an activation mutation of the subunit of the stimulatory GTP-binding protein Gs (Vallar et al, 1987). In these tumors, the mutations that replace amino acids in either position 201 or position 227 of $G\alpha_s$ consititutively activate the $G\alpha_s$ protein and continuously stimulate the adenylyl cyclase signaling pathway, which in turn increases GH secretion and growth of somatotrophs (Landis et

Corresponding to: Seungjoon Park, Department of Pharmacology, Kyunghee University College of Medicine, 1 Hoiki-dong, Dongdaemun-ku, Seoul 130-701, Korea. (Tel) 02-961-0913,0281 (Fax) 02-968-0560 E-mail: sj0915@chollian.net

al, 1989).

Taken together, these findings prompted us to hypothesize that $G\alpha_s$ gene mutation may suppress the expression of TRH-R gene. In order to test our hypothesis, we investigated whether hTRH-R gene expression is lower in human GH-secreting pituitary adenomas with $G\alpha_s$ mutation than in tumors without the mutation and whether rTRH-R gene expression is decreased by expression of the mutant $G\alpha_s$ in a rat GH-secreting cell line.

METHODS

Cell culture

A rat GH-secreting adenoma cell line, GH3, was grown to about 80% confluency in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum. Cells were treated with 50 μM forskolin and 100 μM isobutylmethylxanthine (IBMX), 1 μg/ml cholera toxin, or 1 nM GHRH for 4 h with or without cycloheximide (30 μg/ml).

Transfection

Transfection was performed as described previously (Tian et al, 1994). Cells were seeded into 60-mm tissue culture dishes (1.5×10^6 cells/dish). One day later they were transfected by lipofectin-mediated protocol, as follows: after rinsing with serum-free DMEM, each dish was incubated 6 h at 37°C with 3 ml of Opti-MEM containing 20 μg of Lipofectin plus the indicated plasmids. We used the plasmids pRc/CMV (control), pRc/CMV-α_s (plasmid expressing wild-type $G\alpha_s$), and pRc/CMV-Q227L-α_s (plasmid expressing mutant-type $G\alpha_s$). These plasmids were generous gift from Dr. Joseph P. Pieroni (Department of Pharmacology, Mount Sinai School of Medicine, New York, USA).

Endocrine tests in acromegalic patients

Eight acromegalic patients with GH-secreting pituitary tumors were enrolled. No one had a previous treatment for acromegaly, and had been on medication for the pituitary tumors for at least five months prior to this study. This study was approved by Kyungheee University Hospitals Ethics Committee. Informed consent was obtained from each patient before the study.

For the daytime basal GH secretory profile, blood sampling was done every hour from 08:00 to 14:00. For the paradoxical GH response to TRH, TRH was administered intravenously and blood sampling was done every 30 min for 2 h. Sera were immediately separated and serum GH concentration was measured using a commercial immunoradiometric assay kit. The criteria for the response to TRH was a GH level that exceeded the spontaneous daytime peak and increased more than 50% above the baseline value at 30 min after injection of TRH as we reported previously (Yang et al, 1995).

Direct polymerase chain reaction (PCR) sequencing of the $G\alpha_s$ gene

Genomic DNA was extracted from the frozen tumor tissue and peripheral blood leukocytes of each patient as described previously (Sambrook et al, 1989). Nested PCR was performed to amplify and sequence the region between codon 184 and 251 that includes exon 8 and 9 of the $G\alpha_s$ gene. A set of oligonucleotide primers (sense: 5'-GCG CTG TGA ACA CCC CAC GTG TCT-3'; antisense: 5'-CGC AGG GGG TGG GCG GTC ACT CCA-3') was used for the first PCR amplification. Another set of primers (sense: 5'-GTG ATC AAG CAG GCT GAC TAT GTG-3'; antisense: 5'-GCT GCT GGC CAC CAC CAC GAA GAT GAT-3') was used for second PCR reaction. The amplified DNA fragments were confirmed by agarose gel electrophoresis and purified by electroelution and used as template for direct PCR sequencing. The sequencing reactions were performed by using a cyclic sequencing kit (SequiTherm, Epicentre, Boca-Raton, FL, USA). The base change mutation in each case was confirmed by a second PCR amplification of the tumor tissue.

RT-PCR and in vitro transcription

The quantitiation of TRH-R mRNA was performed by RT-PCR and *in vitro* transcription with co-amplification of internal standard as described previously (Horikoshi et al, 1992). Total RNA was extracted by the method of Chomczynski and Sacchi (1987). One μg of total RNA was reverse transcribed by AMV reverse transcriptase. The resulting cDNA products were subsequently amplified with *Taq* DNA polymerase. The amplified PCR products were transcrib-

ed *in vitro* with T7 RNA polymerase using [α-^{32}P]CTP. The transcription mixtures were separated on 4.5% denatured polyacrylamide gel. After the gel was dried, autoradiography was performed. Using the autoradiogram as a guide, the bands on the gel were marked and cut with scissors and they were counted in a liquid scintillation counter.

The primers were constructed based on the sequences of hTRH-R (Yamada et al, 1993), human β-actin (Ponte et al, 1984), rTRH-R (Zhao et al, 1992) and rat β-actin (Nudel et al, 1983). The primer sequences were as follows: hTRH-R: sense; 5'-GCC TCC CCA ACA TAA CAG ACA-3'; antisense; 5'-GTG ACC TGC TTC CTT GAA GAT-3', human β-actin: sense; 5'-GCG GGA AAT CGT GCG TGA CAT-3'; antisense; 5'-GGA AGG AAG GCT GGA AGA GTG-3'; rTRH-R: sense; 5'-ATC TCC CGC AAC TAC TAC TCA-3'; antisense; 5'-GCC AAG CAG GTG TCA TCA AAT-3'; rat β-Actin: sense; 5'-CCC TGT ATG CCT CTG GTC GTA-3'; antisense; 5'-CAC GCA CGA TTT CCC TCT CAG-3'. A T7 RNA polymerase promoter (TAA TAC GAC TCA CTA TA) and GGGAGA transcription initiation sequence were attached to the 5'-end of sense primers so that the amplified cDNA can be converted to RNA.

Calculation of relative expression of TRH-R gene

To calculate the relative expression of TRH-R gene to that of the internal standard β-actin gene, a ratio was determined between the amount of the radiolabeled PCR product within the linear amplification range of TRH-R gene and β-actin gene (Horikoshi et

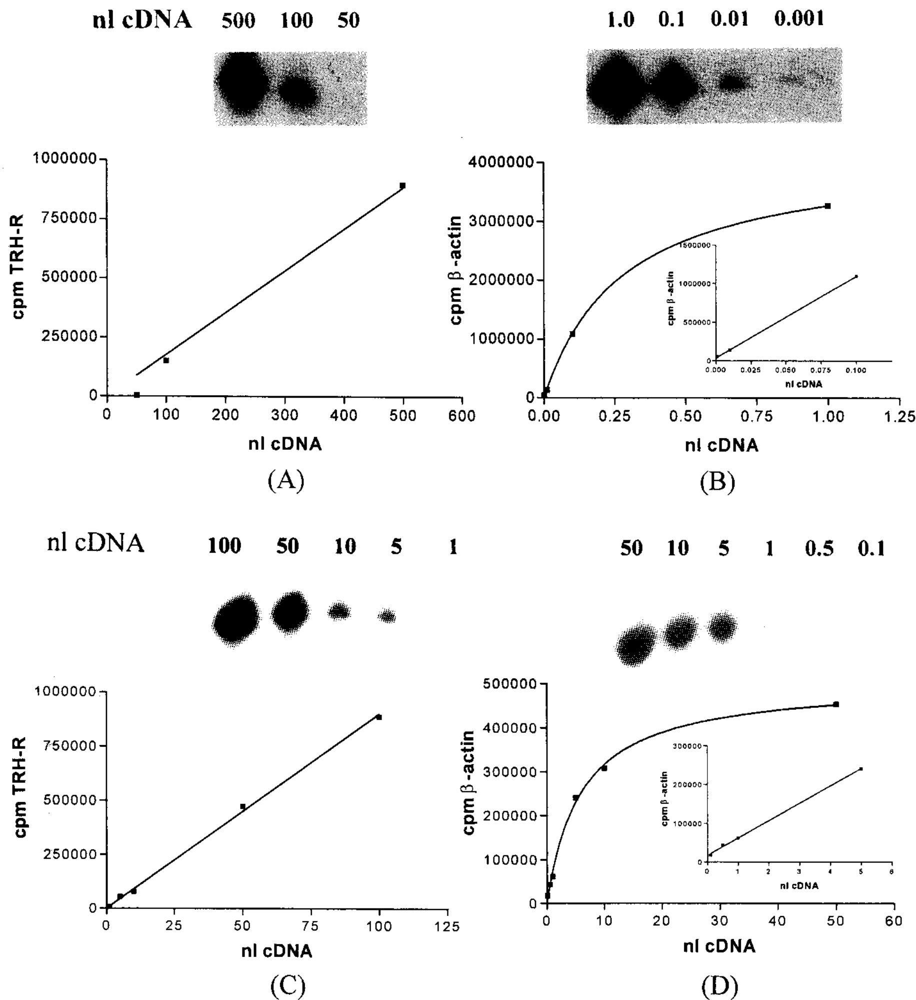

Fig. 1. Optimized linearity regions for PCR amplification of hTRH-R (A), hβ-actin (B), rTRH-R (c), and rβ-actin (B) gene.

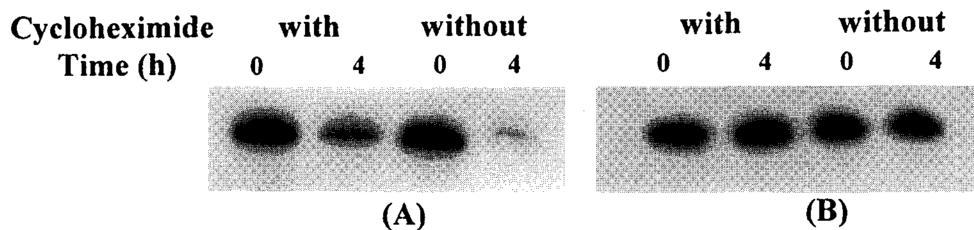

Fig. 2. Effect of forskolin and IBMX on rTRH-R (A) and r-ßactin (B) gene expression in GH3 cells with or without cycloheximide.

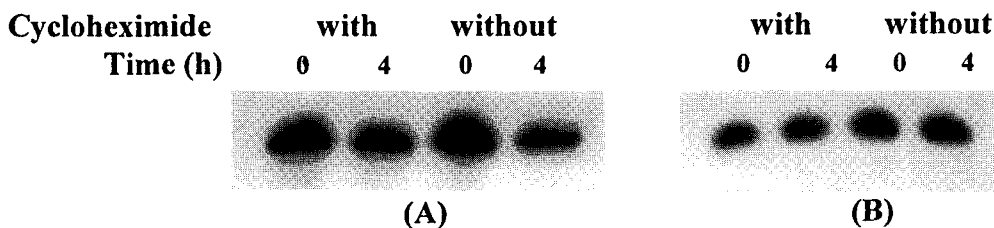

Fig. 3. Effect of cholera toxin on rTRH-R (A) and rß-actin (B) gene expression in GH3 cells with or without cycloheximide.

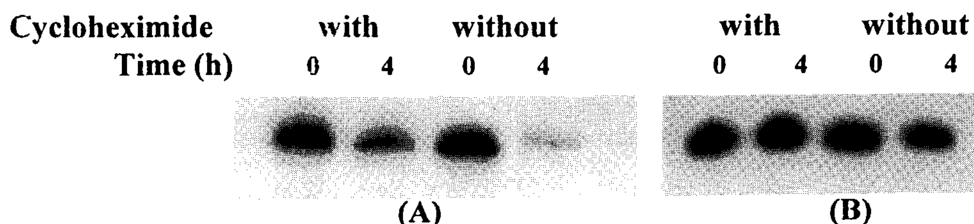

Fig. 4. Effect of GHRH on rTRH-R (A) and rß-actin (B) gene expression in GH3 cells with or without cycloheximide.

al, 1992). Fig. 1 shows the optimized linear regions for the amplification of hTRH-R, rTRH-R, human ß -actin, and rat ß-actin gene.

Statistics

Quantitative data were expressed as mean±standard error of mean (S.E.). Univariate analysis of quantitative data was performed by the Student's t-test (unpaired, two-tailed). Statistical calculations were performed using graphic and statistical software GraphPad Prism™ (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

When GH3 cells were treated with forskolin and IBMX for 4 h, the relative rTRH-R gene expression was decreased by 17.8%. Cholera toxin and GHRH also decreased the rTRH-R transcript 4 h after treatment by 46.0% and 64.9%, respectively. In the presence of cycloheximide, forskolin and IBMX, cholera toxin, and GHRH also decreased rTRH-R gene expression by 39%, 43.7%, and 46.7% at 4 h, respectively (Fig. 2, 3, 4).

Transient expression of a vector expressing mutant type Gαs decreased the relative rTRH-R gene expression by about 50% at 24 h of transfection. On the other hand, wild-type $G\alpha_s$ expression vector did not decreased rTRH-R gene expression significantly (Fig. 5).

The paradoxical response of GH to TRH was found in 4 of 8 (50%) acromegalic patients (Table 1). The transcript of hTRH-R gene was detected in 6 of 8 (75%) tumors. Three of 4 tumors (75%) from the patients with the paradoxical response expressed hTRH-R mRNA, and 3 of 4 tumors (75%) from the patients without the paradoxical response also expressed hTRH-R gene. The relative expression of hTRH-R mRNA in GH-secreting pituitary adenomas from patients with the paradoxical response of GH to TRH did not differ from that in patients without the paradoxical response (0.44±0.34 vs 0.31±0.15) (Fig. 6, 7).

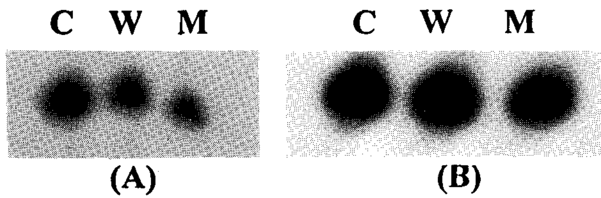

Fig. 5. Effect of pRc/CMV-α_s (W), and pRc/CMV-Q227L-α_s (M) on rTRH-R (A) and rβ-actin (B) gene expression.

Direct PCR sequencing of $G\alpha_s$ between codons 184 and 251 disclosed a mutant allele and a normal allele only at codon 201 in 4 of 8 tumors (Fig. 8). Arginine (CGT) in the normal $G\alpha_s$ protein was replaced with cysteine (TGT). The paradoxical response to TRH was observed in 2 of 4 patients without the mutation, and 2 of 4 patients with the mutation. In the four tumors with $G\alpha_s$ mutation, two tumors did not show any detectable *in vitro* transcribed RT-PCR product and one tumor gave a very small amount of the *in vitro* transcript. However, the remaining one tumor showed a larger amount of the *in vitro* transcript. All tumors without $G\alpha_s$ mutation showed a moderate expression of hTRHR mRNA. The relative hTRH-R gene expression of pituitary adenomas did not differ between the tumors without the mutation and those with mutation (0.64±0.29 vs. 0.11±0.11) (Fig. 6, 7).

DISCUSSION

The paradoxical GH response to TRH is observed

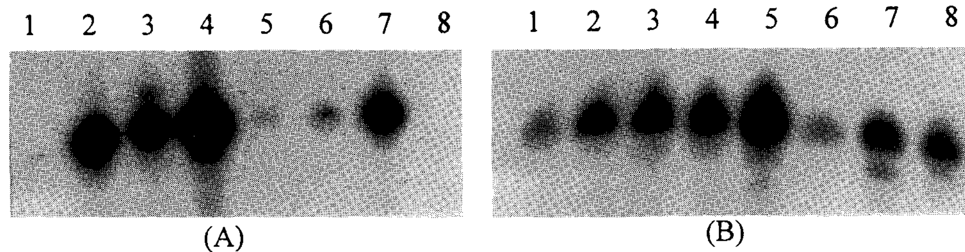

Fig. 6. Expression of gTRH-R (A) and hβ-actin (B) gene in the GH-secreting pituitary tumors.

Table 1. Laboratory findings of the patients with GH-secreting pituitary adenomas

No	Name	Age/Sex	$G\alpha_s$ gene mutation	GH responses to TRH(μg/l)			Relative expression of TRH-R gene($\times 10^{-4}$)
				0 min	30 min	Result	
1	LHS	58/F	201 CGT/TGT	9.0	9.3	−	0.02
2	KSJ	44/F	−	33.4	163.1	+	0.68
3	SKC	43/M	−	16.0	26.3	−	0.32
4	KSH	37/M	−	183.0	170.3	−	1.43
5	SMH	39/F	201 CGT/TGT	9.0	5.6	−	0.00
6	KJS	37/F	−	53.0	335.8	+	0.12
7	JYN	37/M	201 CGT/TGT	30.8	297.2	+	0.43
8	JBH	63/F	201 CGT/TGT	17.7	421.7	+	0.00

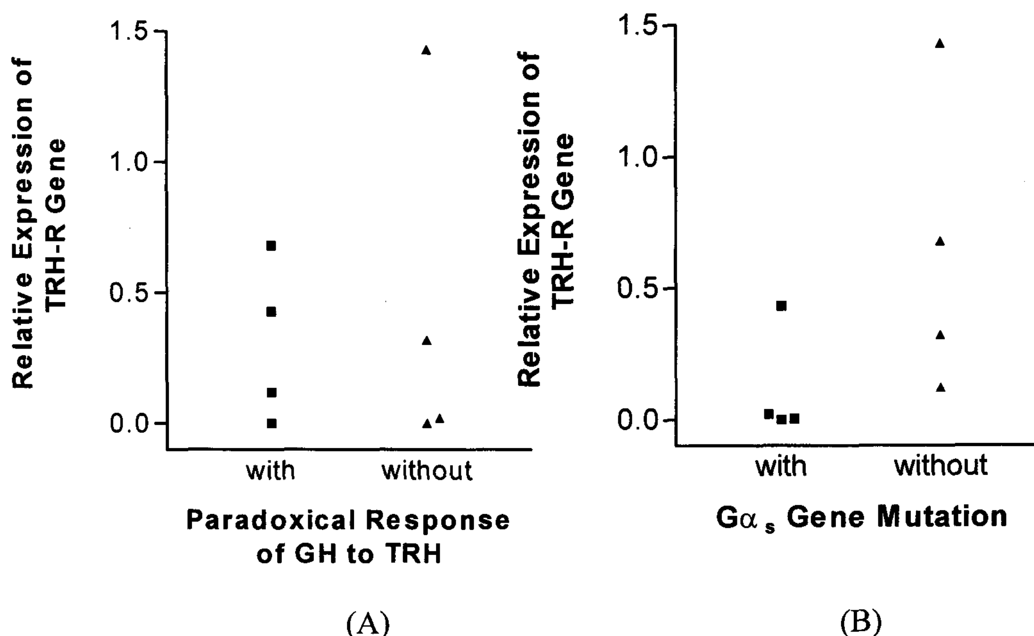

Fig. 7. Relative TRH-R mRNA levels in GH-secreting pituitary adenomas with or without the paradoxical of GH to TRH (A) and with or without the $G\alpha_s$ gene mutation (B).

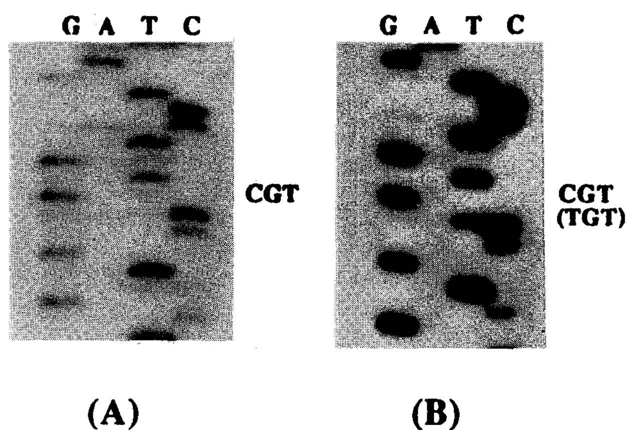

Fig. 8. Point mutations at codon 201 of $G\alpha_s$ gene in GH-secreting tumors. (A) Direct PCR sequencing of the genomic DNA from the peripheral blood leukocytes shows only normal allele CGT. (B) A mutant allele TGT (Cys) and a normal allele were found in the genomic DNA prepared from the tumor.

in a considerable portion of acromegalic patients. Although it was suggested recently that increased GHRH level may mediate the paradoxical response (Hulting et al, 1992), the expression of TRH-R, presumably from the dedifferentiation of tumor cell membrane, has been proposed as a mechanism for the paradoxical response for decades. The hTRH-R cDNA, recently cloned from a GH-secreting pituitary adenoma, was revealed to be identical to that from normal human pituitary gland (Yamada et al, 1993). It supports the possibility that the paradoxical response to TRH is mediated by hTRH-R. However, a recent study demonstrated that hTRH-R mRNA was expressed in the pituitary adenomas not only from acromegalic patients with the paradoxical response of GH to TRH but also from those without the paradoxical response (Kaji et al, 1995). In this study, we also confirmed this finding.

The discrepancy between the paradoxical response and the expression of TRH-R gene suggests at least two possibilities. Firstly, the expression of hTRH-R gene is not enough to elicit the paradoxical response. Other factor(s) in the TRH signal transduction pathway, including the coupled G protein, may be involved in determination of the paradoxical response to TRH. The paradoxical response of patient 8, even though no hTRH-R was detected, suggests the other factor(s) is even more important. Secondly, the transcript detected by RT-PCR may not reflect the normal functioning hTRH-R. For example, an alternative splicing of human GHRH receptor, which in turn produces a truncated receptor, was reported recently (Hashimoto et al, 1995). This possibility needs to be

investigated in the two patients who expressed hTRH-R gene without presenting the paradoxical response.

The results from our *in vitro* study also confirmed the previous report that demonstrated that rTRH-R gene expression in GH3 cells was decreased by agents which elevate intracellular cAMP (Fujimoto and Gershengorn, 1991). The effects of the agents were not affected by the presence of cycloheximide, indicating that all agents affect the transcription of TRH-R gene. TRH is known to initiate some of its effects by interacting with its receptor on the cell surface and that the TRH-R is coupled to G proteins (Hinkle, 1989; Sharif, 1989). The decreased transcription by cAMP suggests a negative feedback between cAMP and TRH-R, and indirectly supports the possibility that $G\alpha_s$ gene mutations suppress TRH-R gene expression.

Somatic mutations of $G\alpha_s$ gene in GH-secreting pituitary tumors have been reported in 30~40% of acromegalic patients (Spada et al, 1992; Yang et al, 1996). Mutations in these tumors replaced Arg 201 (wild-type codon CGT) with either Cys (TGT), His (CAT) or Ser (AGT). And the other mutation replaced Gln 227 (wild-type codon CAG) with Arg (CGG). The mutated $G\alpha_s$ is unable to hydrolyze GTP normally, which results in the activation of adenylyl cyclase and the increase of intracellular cAMP levels for a longer period time.

It was demonstrated that cultured cells expressing mutant-type $G\alpha_s$ had higher cAMP levels than those with the wild-type (Gaiddon et al, 1994). In this study, we demonstrated for the first time that the transient expression of the mutant $G\alpha_s$ gene (Q227L) in GH3 cells, which is known to inhibit the GTPase activity to the same degree as much as R201C (Landis et al, 1989), also decreased the transcription of rTRH-R gene to a similar extent induced by the above cAMP-elevating agents. This finding supports our hypothesis that $G\alpha_s$ gene mutations suppress TRH-R gene expression.

However, in this study, we could not demonstrate that the mRNA expression of hTRH-R in GH-secreting adenomas with the $G\alpha_s$ gene mutation is lower than that in tumors without the mutation. The discordance between this finding and the result from *in vitro* experiment can not be explained clearly. At least two possibilities remain to be investigated. Firstly, a limited number of subjects may not be able to provide a statistical significance. The hTRH-R transcript could hardly be detected in 3 of 4 tumors with $G\alpha_s$ mutation, whereas it was readily detectable in all tumors without the mutation. However, the wide variation of the amount of hTRH-R gene transcript may attribute to the negative result. Secondly, the possibility can not be excluded that the suppression of TRH-R gene expression induced by acute increase of cAMP may not occur in some tumors for unknown reason, such as the tumor of patient 7. The tumor of patient 7, in fact, showed a higher expression of hTRH-R gene.

The present study suggests that the expression of hTRH-R gene is not likely to be a main determinant for the paradoxical response of GH to TRH, and that $G\alpha_s$ gene mutation tends to suppress the gene expression of hTRH-R in GH-secreting adenoma although not statistically significant. It also suggests a certain predisposing factor(s) other than $G\alpha_s$ gene mutation may play an important role in determining the expression of TRH-R.

ACKNOWLEDGEMENT

This study is suppoted by the academic research fund of Ministry of Education, Republic of Korea (1997).

REFERENCES

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal Biochem* 162: 156−159, 1987

Fujimoto J and Gershengorn MC. Evidence for dual regulation by protein kinases A and C of thyrotropin-releasing hormone receptor mRNA in GH3 cells. *Endocrinology* 129: 3430−3432, 1991

Gaiddon C, Boutillier A-L, Monnier D, Mercken L, Loeffler J-P. Genomic effects of the putative oncogene Gs; Chronic transcriptional activation of the c-fos proto-oncogene in endocrine cells. *J Biol Chem* 269: 22663−22671, 1994

Hashimoto K, Koga M, Motomura T, Kasayama S, Kouhara H, Ohnishi T, Arita N, Hayakawa T, Sato B, Kishimoto T. Identification of alternatively spliced mes senger ribonucleic acid encoding truncated growth hormone-releasing hormone receptor in human pitui tary adenomas. *J Clin Endocrinol Metab* 80: 2 993−2939, 1995

Hinkle PM. Pituitary TRH receptors. *Ann NY Acad Sci* 553: 176−187, 1989

Horikoshi T, Daneberg KD, Stadlbauer THW, Volkenandt M, Shea LCC, Aigner K, Gustavsson B, Leichman L, Frosing R, Ray M, Gibson NW, Spears CP, Daneberg PV. Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in hhuman tumors using the polymerase chain reaction. *Cancer Res* 52: 108–116, 1992

Hulting AL, Theodorsson E, Werner S. Thyrotropin-releasing hormone increases serum levels of growth hormone-releasing hormone and growth hormone in patients with acromegaly. *J Int Med* 232: 229–235, 1992

Irie M and Tsushima T. Increase of serum growth hormone concentration following thyrotropin-releasing hormone injection in patients with acromegaly and gigantism. *J Clin Endo Metab* 35: 97–99, 1972

Kaji H, Xu Y, Takahashi Y, Abe H, Tamaki N, Chihara K. Human TRH receptor messenger ribonucleic acid levels in normal and adenomatous pituitary: analysis by the competitive reverse transcription polymerase chain reaction method. *Clin Endocrinol* 42: 243–248, 1995

Landis CA, Masters SB, Spada A, Pace AM, Bourne HR, and Vallar L. GTPase inhibiting mutations activate the chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. *Nature* 340: 692–696, 1989

Nudel U, Zakut R, Shani M, Neuman S, Levy Z, Yaffe D. The nucleotide sequence of the rat cytoplasmic beta-actin gene. *Nucleic Acids Res* 11: 1759–1771, 1983

Ponte P, Ng S-Y, Engel J, Gunning P, Kedes L. Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. *Nucl Acids Res* 12: 1687–1696, 1984

Sambrook J, Fritsch EF, Manniatis T. Molecular Cloning, A Laboratory Manual. 2nd ed. New York, Cold Springer Harbor Laboratory, 1989

Sharif NA. Quantitative autoradiography of TRH receptors in discrete brain regions of different mammalian species. *Ann NY Acad Sci* 553: 147–175, 1989

Spada A, Vallar L, Faglia G. G protein oncogene in pituitary tumors. *Trends Endocrinol Metab* 3: 355–360, 1992

Tian J, Chen J, Bancroft C. Expression of constitutively active Gαs-subunits in GH3 pituitary cells stimulates prolactin promoter activity. *J Biol Chem* 269: 33–36, 1994

Yamada M, Monden T, Satoh T, Satoh N, Murakami M, Iriuchijima T, Kakegawa T, Mori M. Pituitary adenomas of patients with acromegaly express thyrotropin-releasing hormone receptor messenger RNA: Cloning and functional expression of the human thyrotropin-relleasing hormone receptor gene. *Biochem Biophys Res Commun* 195: 737–745, 1993

Yang I, Park S, Woo J, Kim S, Kim J, Kim Y, Choi Y. Characteristics of gsp-positive growth hormone-secreting pituitary tumors in Korean acromegalic patients. *Eur J Endocrinol* 134: 720–726, 1996

Yang IM, Woo JT, Kim SW, Kim JW, Kim YS, Choi YK. Characteristics of acromegalic patients with a good response to octreotide, a somatostatin analogue. *Clin Endocrinol* 42: 295–301, 1995

Vallar L, Spada A, Giannattasio G. Altered Gs and adenylate cyclase activity in human GH-secreting pituitary adenomas. *Nature* 330: 566–568, 1987

Zhao D, Yang J, Jones KE, Gerald C, Suzuki Y, Hogan PG, Chin WW, Tashijan AH Jr. Molecular cloning of a complementary deoxyribonucleic acid encoding the thyrotropin-releasing hormone receptor and regulation of its messenger ribonucleic acid in rat GH cells. *Endocrinology* 130: 3529–3536, 1992